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Polyclonal and Monoclonal Antibodies for the Specific Detection of the Herbicide Acifluorfen and Related Compounds

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Abstract: Polyclonal and monoclonal antibodies reactive with acifluorfen (AF) were prepared by the immunization of, respectively, rabbits and mice with AF-bovine serum albumin conjugates. The reactivities of polyclonal antibody and three monoclonal antibodies (AF 9-1, AF 51-5 and AF 75-144) were examined in an indirect competitive enzyme-linked immunosorbent assay (C-ELISA). The polyclonal antibody reacted with AF at concentrations of 1·5 to 800 μg litre^{-1}, while the monoclonal antibodies reacted with AF at concentrations of 3 to 24 μg litre^{-1} for AF 9-1, 1·5 to 12 μg litre^{-1} for AF 51-5 and 12 to 48 μg litre^{-1} for AF 75-144. In the presence of up to 40% methanol in C-ELISA, the monoclonal antibodies, particularly AF 75-144, were less affected in their reactivities with AF than was the polyclonal antibody. Moreover AF 9-1 and AF 51-5 specifically reacted with acifluorfen-methyl and oxyfluorfen, while AF 75-144 reacted with chlornitrofen which did not react with the other antibodies. These results indicated that the antibodies are useful for the assay of AF and its related compounds.

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1 INTRODUCTION

The herbicide acifluorfen (AF), 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid, is in use today for the control of many broad-leaf weeds in soybean, peanut and rice.[1,2] It was reported that, in the field, AF was rapidly metabolized in crop plants and also readily decomposed by ultraviolet light with half-lives of 30–60 days in water and in soil.[3] However, monitoring of AF residues is important for environmental surveillance because of its long-term use. Analysis of AF residues was performed by gas-chromatography[4] and high-performance liquid chromatography,[5,6] although these methods were time-consuming and labour-intensive. Therefore, development of a rapid and convenient method is required for analysis of a large number of environmental samples including water, soil and crops.

Since many papers have been published on immunoassays of herbicide, insecticide and fungicide chemicals in recent years,[7–9] we attempted to develop immunoassays for detection of AF residues. Both polyclonal and monoclonal antibodies were raised against AF and then characterized in their reactivities with AF and its related chemicals in an enzyme-linked immunosorbent assay (ELISA) and in an indirect competitive enzyme-linked immunosorbent assay (C-ELISA). Particularly, effects of organic solvents in C-ELISA were examined, since residues are usually extracted with organic solvents from environmental samples.

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2 EXPERIMENTAL METHODS

2.1 Chemicals

Acifluorfen was a gift from BASF Japan, Tokyo, Japan. Acifluorfen-methyl (AF-CH_3), oxyfluorfen (OXY) and bifenox (BFX) were gifts of Asahi Chemical Manufacturing Co., Tokyo, Japan. Chlornitrofen (CNP) and chlornitrofen-NH_2 (CNP-NH_2) were obtained from Mitsui-Toatsu Chemicals Inc., Tokyo, Japan. Nitrofluorfen (NF), was a gift from Sumitomo Chemical Co., Ltd, Takarazuka, Japan. Acifluorfen-NH_2 (AF-NH_2) was prepared by reducing a nitro group with iron and acetate, and then the product was confirmed by [^{1}H]NMR analysis.

Bovine serum albumin (BSA) was purchased from Sigma Chemical, MO, USA. Iscove's modified Dulbecco's medium (IMEM) was purchased from ICN Biomedicals, CA, USA. Fetal bovine serum (FBS) was obtained from Xavier Investments, Wacol, Australia. Avid AL gel was from BioProbe International, CA, USA. Peroxidase-conjugated goat anti-mouse IgG antibodies (POD conjugated anti-mouse IgG) and peroxidase-conjugated goat anti-rabbit IgG antibodies (POD conjugated anti-rabbit IgG) were products of Organon Teknika, NC, USA. A biotinylated anti-mouse Ig subclass collection was from Amersham plc, Buckinghamshire, UK. Alkaline phosphatase-conjugated avidine was from Dakopatts, Glostrup, Denmark. Microplates with 96-wells from Corning, NY, USA were used for cell culture and these from Costar, MA, USA were used for ELISA and C-ELISA. All other chemicals and reagents were of analytical grade.

2.2 Preparation of AF-BSA conjugates

AF was coupled covalently with BSA by the mixed-anhydride method.[10] AF (70 μmol) dissolved in anhydrous, 1,4-dioxane (4 ml) was mixed with *N*-methylmorpholine (1·0 mmol) and kept for 20 min at 10°C, followed by the addition of isobutyl chlorocarbonate (0·3 mmol). The reaction mixture was stirred for 15 min at 10°C, and then was added dropwise to BSA (80 mg) dissolved in water (4·3 ml) which was adjusted to pH 9 with sodium hydroxide (1 M) and kept for 4 h at 4°C to complete the coupling reaction. The reaction mixture including AF-BSA conjugate was then dialysed against phosphate-buffered saline (PBS) overnight at 4°C.

2.3 Preparation of antibodies

2.3.1 Polyclonal antibodies

Three eight-week-old female white rabbits purchased from Saito Ikuseijo (weight about 1 kg) were immunized with AF-BSA conjugate (1·0 mg) dissolved in PBS and emulsified with Freund's complete adjuvant at a final concentration of 0·5 g litre^{-1}. Two millilitres of the antigen emulsion were injected at multiple subcutaneous sites on the back of each animal. Then, the animals were boosted with the same antigen (200 μg) emulsified with Freud's incomplete adjuvant on 30 and 70 days after primary immunization. They were bled through their median ear veins 10 days after the last injection, and sera prepared from their whole blood were precipitated by adding saturated ammonium sulfate solution to final 33% saturation. Precipitates formed were dissolved in PBS and dialysed against the same buffer at 4°C.

2.3.2 Monoclonal antibodies

Five eight-week-old female Balb/C mice purchased from Japan Clea (weight about 25 g) were each immunized with AF-BSA conjugate (100 μg), according to the same schedule as described for polyclonal antibodies. The antigen emulsion was injected subcutaneously on the back of each animal. The animals were boosted with the same antigen (30 μg) 30 and 70 days after primary immunization. Three days after the last injection, one of the immunized mice was provided to prepare hybridomas producing anti-AF monoclonal antibodies.[11] Spleen was removed and splenocytes were fused with P3-X63-AG8.653 myeloma cells[12] by using polyethylene glycol 1500 and then grown selectively in IMEM fortified by 10% FBS, hypoxanthine (100 μM), aminopterin (0·4 μM) and thymidine (16 μM) (HAT medium). Cultured fluids of hybridomas were screened for their binding ability to AF-BSA conjugate in ELISA. Positive hybridomas were cloned by limiting dilution. Clones selected were grown in IMEM fortified by 10% FBS (complete medium) and their cultured fluids were taken to the same ELISA as the above screening. Representative clones were transferred to the culture in IMEM fortified with 2% FBS after they were grown to 10^8 cells in complete medium. Monoclonal antibodies included in their cultured fluid were purified by the aid of Avid AL gel.[13]

2.4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

The antibodies purified were analysed by SDS-PAGE as described by Laemmli.[14] The antibodies were solubilized in SDS-PAGE sample buffer (SDS 10 g litre^{-1} Tris-HCl 62·5 mM, pH 6·8, glycerol 100 g litre^{-1}, bromophenol blue 0·01 g litre^{-1}) and then heated for 5 min at 95°C. The samples were loaded on a gel (separation gel, 7·5%, spacer gel, 4%) and run until the blue dye had moved out of the gel. The gel was stained with 0·1% coomassie brilliant blue in acetic acid + methanol + distilled water (7 + 50 + 43 by volume). After the gel had been destained in acetic acid, methanol and distilled water (7 + 10 + 83 by volume),

the visualized bands of samples were compared with those of standard mouse IgG.

2.5 ELISA with AF-BSA conjugate

ELISA was performed to screen cultured fluids of hybridomas and to titrate dilution rates (concentrations) of the fluids, anti-serum and purified anti-AF antibodies which bound to AF-BSA conjugate at approximately 50% of available sites on the solid phase, abbreviated as 50% binding ability. Microtiter plates with 96 wells were coated with 50 μl of AF-BSA (400 μg litre^{-1}) and left overnight at 4°C. Wells were aspirated off immediately before use. Sites on wells unoccupied by coating antigen were blocked with 250 μl of 10 g litre^{-1} BSA in borate buffered saline (85 mM borate, 150 mM NaCl, pH 8·0; BBS) for monoclonal antibodies or 3% skim milk in BBS for polyclonal antibodies and kept for 1 h at 25°C, followed by washing three times with BBS. Aliquots (100 μl) of 1st antibodies (anti-serum, cultured fluid or purified antibody) diluted in BBS modified with 3 g litre^{-1} BSA for monoclonal antibodies or 0·3% skim milk for polyclonal antibodies were then added to the wells coated with antigen, and the plates were incubated for 1 h at 25°C, followed by washing again. One hundred microlitres of POD conjugated anti-mouse IgG or anti-rabbit IgG diluted to 1 : 1000 with the same buffer as 1st antibodies were then added to the wells and the plate was incubated for 1 h at 25°C. After washing again, 100 μl of the substrate (100 mg litre^{-1}, 3,3′,5,5′-tetramethylbenzidine) solution in 100 mM sodium acetate (pH 5·5)-0·03% hydrogen peroxide were added to the wells. The plates were then incubated for 10 min at 25°C, colour reaction was stopped by adding an equal volume of sulfuric acid (0·5 M), and the absorbance at 450 nm was measured with an automated microplate reader (Bio-tek Instruments, Vermont, USA). All the samples were assayed in triplicate and the assay was also performed in triplicate.

2.6 C-ELISA for AF and its related chemicals

A C-ELISA protocol, which was a modified version of ELISA, was arranged to assay AF and its structurally related chemicals. Microtiter plates with 96 wells were coated with AF-BSA conjugate, followed by blocking, as described with ELISA (Section 2.5). Aliquots (50 μl) of analyte dissolved in BBS supplemented with methanol of appropriate ratio (0·5–80%) were transferred to the wells. An equal volume of antibodies which was two-fold higher than the concentration of 50% binding ability was then added immediately and mixed by tapping. The same buffer as used for the analyte was used for the dilution of antibodies. The plates were incubated for 1 h at 25°C, followed by the procedure as described with ELISA. All the samples were assayed in triplicate, and the assay was also performed in triplicate.

3 RESULTS

3.1 Polyclonal and monoclonal antibodies raised against AF

3.1.1 Polyclonal antibodies

Anti-sera were obtained from three rabbits immunized with the AF-BSA conjugate. About 250 000 to 300 000-fold dilutions of the three sera were sufficient to show 50% binding ability to the AF-BSA conjugate in ELISA. These sera did not react with BSA which was used for AF conjugation. Out of three anti-sera, the serum titrated at the highest dilution rate in ELISA was purified for further examinations. When the polyclonal antibody prepared by ammonium sulfate precipitation was analysed by SDS-PAGE with 7·5% separating gels without 2-mercaptoethanol, the antibody showed a major protein band on SDS-PAGE (data not shown). The 50% binding ability to the AF-BSA conjugate of the purified antibody was determined to be 44 μg litre^{-1} by spectrophotometrical measurement at 280 nm. Therefore, C-ELISA was examined with a two-fold higher concentration of the polyclonal antibody. The reaction of the polyclonal antibody with the AF-BSA conjugate was inhibited by free AF (1 μg litre^{-1}) in C-ELISA as shown in Table 1.

3.1.2 Monoclonal antibodies

Five Balb/c mice were each immunized with the AF-BSA conjugate. Their spleens, were removed, splenocytes were prepared and fused with P3-X63-AG8.653 myeloma cells. Fused cells (hybridomas) were selectively grown in the microplates with 96 wells containing HAT medium. Antibodies secreted into the cultured fluids of hybridomas were screened for their binding ability to the AF-BSA conjugate in ELISA. The culture fluids reactive with BSA were counter-selected by re-screening in ELISA coated with BSA instead of the AF-BSA conjugate. About 400 wells were screened and 23 positive wells were obtained. Hybridomas grown in these wells were cloned by limiting dilution, and 18 out of the positive hybridomas were cloned. The 50% binding ability to the AF-BSA conjugate was varied in the range of 10 to 3000-fold dilutions among the fluids. The reaction of eight out of 18 monoclonal antibodies with the AF-BSA conjugate was inhibited by free AF (1 μg litre^{-1}) in C-ELISA as shown in Table 1. These 18 antibodies were also characterized by means of ELISA using a biotinylated anti-mouse Ig subclass collection and alkaline phosphatase conjugated avidine. These subclasses were IgG1 (12 antibodies), IgG2a (three antibodies) and IgG2b (three antibodies) as shown in Table 1. It was interesting to know that all eight antibodies reactive

TABLE 1
Characteristics and Reactivity with Acifluorfen (AF) of Polyclonal and Monoclonal Antibodies

Antibody	*Subtype*	*AF*[a]	*Antibody*	*Subtype*	*AF*
Polyclonal antibody		+			
Monoclonal antibody			Monoclonal antibody		
AF 9-1	IgG1	+	AF 83-162	IgG1	+
AF 19-11	IgG2a	−	AF 86-182	IgG2a	−
AF 31-87	IgG2b	−	AF 88-15	IgG1	−
AF 40-91	IgG1	−	AF 110-31	IgG1	+
AF 51-5	IgG1	+	AF 135-38	IgG1	+
AF 62-117	IgG2b	−	AF 138-48	IgG1	−
AF 66-135	IgG2a	−	AF 141-38	IgG2b	−
AF 75-144	IgG1	+	AF 149-64	IgG1	+
AF 81-155	IgG1	+	AF 151-67	IgG1	−

[a] + Shows inhibition of AF in the reaction of an antibody with the AF-BSA conjugate and − shows no inhibition of AF in the reaction of an antibody with the AF-BSA conjugate.

with AF were IgG1. Three monoclonal antibodies (AF 9-1, AF 51-5 and AF 75-144) were selected on the basis of reactivity with AF for further examinations. The hybridomas producing those three monoclonal antibodies were cultured in IMEM fortified with 2% FBS, and the antibodies secreted were purified by the affinity chromatography. When the antibodies prepared were analysed by SDS-PAGE, AF 51-5 and AF 75-144 showed a major protein band, while AF 9-1 contained four main bands (data not shown). The concentrations of AF 9-1, AF 51-5 and AF 75-144 on the basis of the 50% binding ability to the AF-BSA conjugate were estimated to be 1·4 μg litre^{-1}, 0·2 μg litre^{-1} and 12·0 μg litre^{-1}, respectively, when measured as described for the polyclonal antibody. Therefore C-ELISA was examined with a two-fold higher concentration of each of these monoclonal antibodies.

3.2 The reactivity of polyclonal and monoclonal antibodies with AF

The reactivity of polyclonal and monoclonal antibodies with AF was assayed in C-ELISA. In Fig. 1, AF concentrations were plotted on the x axis and inhibition rates, calculated as a percentage against an absorbance in the absence of AF, were plotted on the y axis. Inhibition curves of polyclonal and monoclonal antibodies were generated for varying AF concentrations in 0·5% methanol. The curve of the polyclonal antibody was virtually linear in the range of 1·5 to 800 μg litre^{-1} of AF. On the other hand, the inhibition curves of the monoclonal antibodies plotted on the same axis as the polyclonal antibody were fitted smoothly to the expected sigmoid, and their linear ranges were diversified among the antibodies as determined in the range of 3 to 24 μg litre^{-1} (AF-9-1), 1·5 to 12 μg litre^{-1} (AF 51-5) and 12 to 48 μg litre^{-1} (AF 75-144). Thus, these antibodies were found to be different in their reactivities with AF. In addition, the inhibition curves of the monoclonal antibodies for AF showed about 1/70 to 1/130-fold narrower ranges, resulting in the steep curves, as compared with that of the polyclonal antibody. The 50% inhibition of the monoclonal antibodies was at the concentrations of 3·1 μg litre^{-1} (AF 51-5), 19 μg litre^{-1} (AF 9-1) and 35 μg litre^{-1} (AF 75-144) instead of 36 μg litre^{-1} of the polyclonal antibody. Based on these results, the monoclonal antibodies were found to be more reactive with AF than the polyclonal antibody. Particularly, AF 51-5 was the most sensitive in the detection of AF in C-ELISA.

3.3 Effects of organic solvents on the reaction of polyclonal and monoclonal antibodies with AF

Since AF residues are often extracted with organic solvents from environmental and crop samples for analysis, the effects of these solvents were examined in

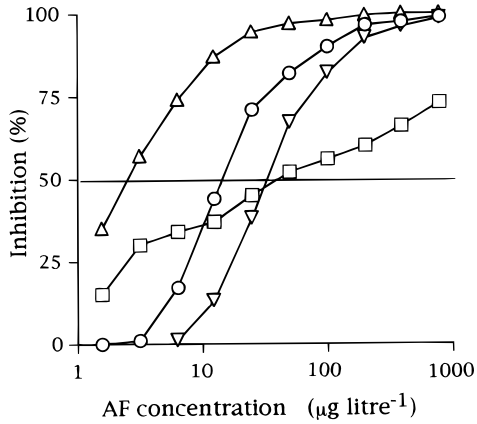

Fig. 1. The reactivity of polyclonal and monoclonal antibodies with acifluorfen: (□) polyclonal antibody, (○) AF 9-1, (△) AF 51-5 and (▽) AF 75-144.

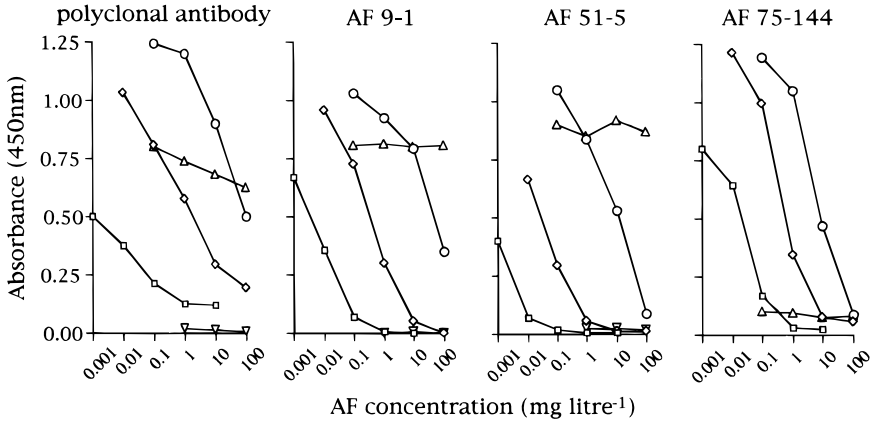

Fig. 2. Effects of methanol on the reaction of each of the antibodies with acifluorfen: (□) 0·5% methanol, (◇) 20% methanol, (○) 40% methanol, (△) 60% methanol and (▽) 80% methanol.

C-ELISA. Practically, methanol seemed to have the least adverse effect on the reaction of the antibodies with AF among various organic solvents examined. Therefore, the effects of methanol were examined by adding it at final concentrations of 0·5 to 80% to the competitive reaction mixture in C-ELISA (Fig. 2). With the polyclonal antibody, the inhibition curves were steeper at higher methanol concentrations, and the reactivity was completely lost in 80% methanol. The effects of methanol on the reaction of the monoclonal antibodies with AF were also examined. When AF 9-1 was assayed for AF in up to 40% methanol, the reactivity was shifted to a lower level and lost completely in 80% methanol. The inhibition curves of AF 51-5 showed a similar pattern to that with AF 9-1. The reactivity of AF 75-144 shifted to lower parallels depending on the increased methanol concentration, to a lesser extent than with the other monoclonal antibodies. The reactivity was completely lost in 60% methanol. Based on the results, it was found that the polyclonal and monoclonal antibodies reacted with AF in the presence of methanol at up to 40% concentrations in C-ELISA. The monoclonal antibodies were less affected by methanol in reactivity with AF in C-ELISA as compared with the polyclonal antibody. Particularly, AF 75-144 was the most reactive with AF in up to 40% methanol.

3.4 Effects of methanol on the reaction of the antibodies with AF-related compounds

The reactivities of the polyclonal and monoclonal antibodies with AF and its structurally related compounds shown in Fig. 3 were assayed in C-ELISA. Particularly, the effects of methanol were examined at final 0·5% and 40% concentrations in the competitive reaction mixture. The results are shown as IC_{50} values in Table 2. The polyclonal antibody showed similar IC_{50} values with

TABLE 2
The Reactivity of Each of the Antibodies with Acifluorfen and its related compounds in the Presence of 0·5% and 40% Methanol

		IC_{50} (*log* nM)							
		Poly Ab[b]		*AF 9-1*		*AF 51-5*		*AF 75-144*	
Compound[a]	*Abbreviation*	*0·5%*[c]	*40%*[d]	*0·5%*	*40%*	*0·5%*	*40%*	*0·5%*	*40%*
1	AF	2·0	4·8	1·8	4·8	1·0	4·1	2·0	4·1
2	AF-NH_2	2·9	5·0	2·9	>5·5	2·4	>5·5	>3·5	>5·5
3	AF-CH_3	2·1	3·9	0·5	2·8	0·3	2·7	1·8	2·5
4	OXY	3·0	4·1	0·8	2·8	0·6	3·0	2·8	4·5
5	NF	>3·5	4·1	1·6	3·9	1·0	3·3	2·4	4·5
6	BFX	3·1	4·7	1·5	4·3	1·1	4·1	1·8	3·4
7	CNP	>3·2	>5·5	>3·2	>5·5	>3·2	>5·5	2·7	4·4
8	CNP-NH_2	>3·5	>5·5	>3·5	>5·5	>3·5	>5·5	>3·5	>5·5

[a] See Figure 3.
[b] Polyclonal antibody.
[c] 0·5% methanol in the competitive reaction mixture.
[d] 40% methanol in the competitive reaction mixture.

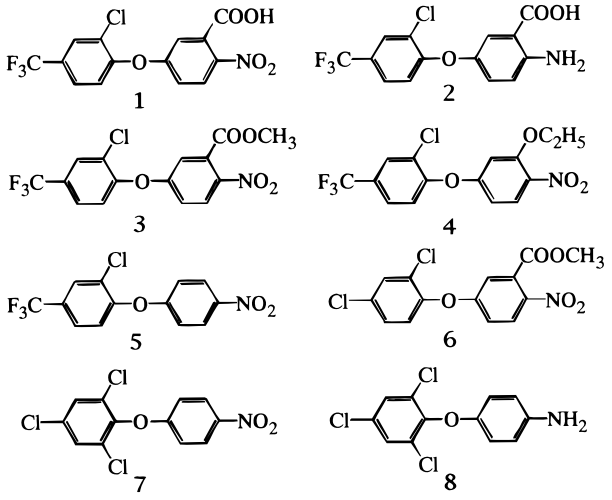

Fig. 3. Structures of compounds discussed. (**1**) Acifluorfen, (**2**) Acifluorfen-NH_2, (**3**) Acifluorfen-methyl, (**4**) Oxyfluorfen, (**5**) Nitrofluorfen, (**6**) Bifenox, (**7**) Chlornitrofen and (**8**) Chlornitrofen-NH_2.

AF and AF-CH_3 in 0·5% methanol, while with AF-NH_2, OXY and BFX, higher IC_{50} values were found than with AF. These reactivities were reduced and showed higher IC_{50} values in 40% methanol as compared with these in 0·5% methanol.

The reactivity of the monoclonal antibodies with AF and its related compounds was also examined in C-ELISA in the presence of 0·5% and 40% methanol. Both AF 9-1 and AF 51-5 specifically reacted with AF-CH_3 and OXY and showed lower IC_{50} values than that of AF in 0·5% methanol, whereas AF, NF and BFX showed larger IC_{50} values with both antibodies. Both monoclonal antibodies showed larger IC_{50} values for AF-NH_2 than for AF, although the reactivity was lost in 40% methanol.

AF 75-144 differed from the other monoclonal antibodies, being less reactive with AF-CH_3 and OXY in 0·5% methanol. However, AF 75-144 cross-reacted with CNP, although the other antibodies did not. In addition, AF 75-144 did not react with AF-NH_2. The reactivity of AF 75-144 was also reduced in 40% methanol, although it still reacted with CNP even at higher IC_{50} values.

Based on these results, it was found that the monoclonal antibodies were more reactive than the polyclonal antibody and were different in their reactivity with AF and its related compounds in C-ELISA. Both AF 9-1 and AF 51-5 specifically reacted with AF-CH_3 and OXY, while AF 75-144 reacted with CNP, which was not reactive with the other antibodies.

4 DISCUSSION

We prepared both polyclonal and monoclonal antibodies derived from AF-BSA conjugate. The polyclonal antibody reacted with AF at a wide range of the concentrations, while the reactivity of the monoclonal antibodies was higher at lower concentrations of AF. Therefore, the monoclonal antibodies seemed to be useful for accurate detection of AF as compared with the polyclonal antibody as reported with the herbicide picloram.[15]

Since AF residues in environment and crop samples are often extracted with organic solvents, we examined the effects of methanol in C-ELISA. The antibodies reacted with AF in up to 40% methanol, although the reactivities of the antibodies with AF were reduced in 40% methanol. These results suggested that the antibodies were useful for assay of AF residues in methanol extracts of samples, resulting in simplification of assay steps.

When 2 mg kg^{-1} of AF was added to soil samples obtained from the paddy field, the recovery of AF in the methanol extracts was 105% in C-ELISA. Also, on addition of 8 μg $litre^{-1}$ of AF to water samples obtained from some paddy fields, the recovery of AF was 89–98% (unpublished observation). Both AF 9-1 and AF 51-5 were sensitive for detecting AF-CH_3 and OXY in 0·5% methanol, and may be useful for detection of AF-NH_2 in differential assay in both 0·5% and 40% methanol, since both antibodies reacted with AF-NH_2 in 0·5% methanol but not in 40% methanol. In addition, AF 75-144 seemed to be useful for detection of CNP, since the antibody did not react with CNP-NH_2.

The antibodies seemed to react with a specific part and/or orientation of the compounds. Actually, in 0·5% methanol, the antibodies may recognize both the nitro group and the carboxyl group of AF, which was used for conjugation with BSA, since the antibodies reacted with AF, AF-CH_3, OXY, BFX and NF. The trifluoromethyl group of AF may be also important for reaction with the antibodies, although BFX reacted favourably with the antibodies.

Polyclonal antibodies have been used for assay of biopolymers and hapten. Monoclonal antibodies have been used for the assay of biopolymers since Köhler and Milstein's invention in 1975.[16] However, the application of monoclonal antibodies to the assay of herbicide residues[15,17–19] was quite limited as compared with polyclonal antibodies. Although with biopolymers there is a risk of alteration of antigenicity by mutation, herbicide chemicals show a constant antigenicity provided that the chemicals are not metabolized. Therefore the herbicide chemicals may keep constant antigenicity against the monoclonal antibodies.

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